AD_____

Award Number: DAMD17-03-1-0749

TITLE: Physical Characterization of a Highly Infectious Monodisperse Preparation of TSE Infectivity as a Substrate for Diagnostic Development

PRINCIPAL INVESTIGATOR: Robert G. Rohwer, Ph.D.
Andrew G. Timmes, Ph.D.
Luisa L. Gregori, Ph.D.
Irina Alexeeva, M.D., Ph.D.

CONTRACTING ORGANIZATION: Baltimore Research and Education Foundation
Baltimore, MD 21201

REPORT DATE: September 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-09-2007	Final	1 Sep 2003 – 31 Aug 2007

4. TITLE AND SUBTITLE

Physical Characterization of a Highly Infectious Monodisperse Preparation of TSE Infectivity as a Substrate for Diagnostic Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0749

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert G. Rohwer, Ph.D.,
Andrew G. Timmes, Ph.D.
Luisa L. Gregori, Ph.D.
Irina Alexeeva, M.D., Ph.D.
Email: rrohwer@umaryland.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baltimore Research and Education Foundation
Baltimore, MD 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purification and characterization of the infectious agent that causes transmissible spongiform encephalopathies would be a breakthrough in the development of diagnostic tests and treatments for the victims of these fatal neurological diseases. Using a preparation of highly dispersed, nanofiltered scrapie infected brain homogenate, we have developed an ultracentrifugation procedure for the purification of dispersed, small infectious particles. The design of this protocol was guided by our determinations of the buoyant density and sedimentation constant of scrapie infectivity and its only known marker, PrPres. We are actively pursuing scaled up pathways to achieve the original plan of this project, which is to use buoyant density, sedimentation rate, and other biochemical characteristics to concentrate, purify, and study the scrapie infectious agent.

15. SUBJECT TERMS
Purification, Centrifugation, Filtration, Diagnostics, Physical Properties

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	27	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents:

Introduction…….……..……4

Body……..4

Key Research Accomplishments…….…….13

Reportable Outcomes.....14

Conclusions…….…….15

INTRODUCTION:

Purification and characterization of the infectious agent that causes transmissible spongiform encephalopathies would be a breakthrough in the development of diagnostic tests and treatments for the victims of these fatal neurological diseases. Using a preparation of highly dispersed, nanofiltered scrapie infected brain homogenate, we have developed an ultracentrifugation procedure for the purification of dispersed, small infectious particles. The design of this protocol was guided by our determinations of the buoyant density and sedimentation constant of scrapie infectivity and its only known marker, PrP^{res}. We are actively pursuing scaled up pathways to achieve the original plan of this project, which is to use buoyant density, sedimentation rate, and other biochemical characteristics to concentrate, purify, and study the scrapie infectious agent. The techniques used are unbiased in their ability to isolate infectious particles, whether they are purely proteinaceous or contain other factors.

BODY:

The Aims referenced below are taken directly from the approved Statement of Work and approved Revised Statement of Work.

Aim I. To measure the sedimentation constant and the buoyant density of the infectivity in the monodisperse preparation and to compare these values to those of PrP^{res} in the preparation. The values obtained for the infectivity will be used to guide the purification procedures in Aim III.
Buoyant density

As projected in our first specific aim, the buoyant density of the infectivity has been determined in a CsCl density gradient equilibrium ultracentrifugation experiment. Two peaks with values of 1.24 +/- 0.01 and 1.29 +/- 0.01 g/ml were identified. These matched the peak densities of PrP^{res}, the only known marker for TSE infectivity. Mysteriously, the observation of two PrP^{res} peaks was not reproducible using subsequent lots of nanofiltrate (also called monodisperse preparation). A second CsCl density gradient experiment with bioassays has been carried out using a new method that consistently produces just one peak of PrP^{res} at 1.29 +/- 0.01 g/ml. Surprisingly, two peaks of infectivity at 1.23 +/- 0.01 and 1.29 +/- 0.01 g/ml were detected (Figure 1), matching the gradient run in 2004. The ability to create a fraction that is enriched in infectivity (at 1.23 g/ml) relative to PrP^{res} was an exciting development. Because the data showing two peaks was based on incubation time measurement method, we re-inoculated the peak fractions and the trough fraction between them in endpoint dilution titrations, a more accurate, though time-consuming method. After 300 days post inoculation (dpi), the results are not yet clear (Table 1). Calculation of the titers from the current numbers of normal and scrapie-affected animals indicates a second peak, although it is smaller than the peak which coincides with the PrP^{res} peak.

The trend of the re-inoculation data, however, supports the projection that the final titers will not demonstrate a second peak enriched in infectivity relative to PrP^{res}. The titration of Fraction 14, the 1.29 g/ml peak has infections down to the 10^{-7} dilution. Fraction 16, the trough fraction, has infections down to the 10^{-6} dilution. Fraction 18, the 1.23 g/ml peak fraction only has infections down to the 10^{-5} dilution. The descending order of last dilution with an infection,

from -7 to -6 to -5, is supportive of a single infectious peak. As the bioassay continues (until 540 dpi), we will see whether more infections develop in the higher dilutions of the 1.23 g/ml peak fraction and what the final titers are by Reed-Muench calculation. If the enriched peak is verified, we intend to exploit this finding for increasing the purity of infectivity.

A major difference between our efforts and previous purifications of scrapie infectivity (fibril preps) is our commitment to maintaining the infectious particles in a dispersed form. We therefore developed the Filtration Assay, which uses inexpensive 0.1 µm Anotop filters to assess the aggregation state of PrP^{res}. PrP^{res} from nanofiltrate will pass through the filters 100%, but after incubation in high concentrations of CsCl, it was blocked, indicating an increase in particle diameter (Figure 2). Interestingly, the aggregation caused by CsCl does not result in a great loss of infectivity. Aggregated particles are expected to have lower apparent infectivity, but our testing showed that this was not the case (Table 2). We suspect that the aggregation is partially reversed by dilution and ultrasonication, which we routinely employ prior to inoculation. An alternative density media, sucrose, did not cause this aggregation. We therefore explored the use of sucrose for determination of buoyant density, sedimentation constant, and large scale purification.

The buoyant density of PrP^{res} and infectivity were determined using sucrose dissolved in D_2O as the density medium. The use of deuterium in place of hydrogen increases the density of the solution without increasing the viscosity, which slows the sedimentation of particles. The substrate used was not unpurified nanofiltrate, as in the CsCl experiments, but pooled fractions from the Step Gradient Two protocol to be described under Specific Aim II in this report. Non-PrP protein in this pool is reduced by approximately 99% compared to the nanofiltrate. 11 ml of Step Gradient Two Pool were loaded onto a sucrose gradient consisting of 6 ml of 40% sucrose, 6 ml of 50% sucrose, and 10 ml of 60% sucrose. After spinning for 42 hours at 32,000 rpm (175,000 g_{max}) in the SW 32 Ti rotor at 20°C, the sucrose had formed a continuous gradient (Figure 3). Fractions were collected from the bottom of the tube and assayed for PrP and ferritin by western blots and by incubation time bioassay for infectivity (Figure 3, Figure 4). Infectivity and PrP^{res} co-purified in a wide peak at a density of 1.25-1.26 g/ml. Ferritin also formed a broad peak, but the density at the highest concentration was 1.30 g/ml. The peak PrP^{res} fraction, 17, was inoculated for endpoint dilution titration, as was the peak of ferritin, fractions 11 and 12 pooled. The bioassay is ongoing, but so far indicates that infectivity is proportionate to PrP^{res} content and ferritin (at least the majority of it) is irrelevant to infectivity (Table 3). Because of their breadth, the two peaks overlapped somewhat and a small amount of ferritin was found in the PrP^{res} peak. This study both provided us with a physical property of PrP^{res} and demonstrated a method for greatly increasing the purity of the small, dispersed, infectious particles.

It is not surprising that both PrP^{res} and endogenous ferritin have heterogeneous densities, in contrast to the uniform virions of conventional viruses. Evidence for variable density of the scrapie agent has been observed for decades, and indeed, this heterogeneity obstructed early purification attempts. The explanation for this variability, in the context of the prion hypothesis, still awaits elucidation. Ferritin particles can contain a wide range of numbers of iron atoms from zero to 4000 and may also sequester other elements such as phosphorus. Particles with greater iron content possess higher densities. It is reasonable to suppose that the lighter ferritin particles that co-purify with PrP^{res} are closer to the state of apoferritin, which is devoid of iron.

There is no evidence that the majority of endogenous ferritin in hamster brain is related to scrapie infectivity. According to our incubation time bioassay, the ferritin peak has infectivity

in proportion to its PrP^{res} content. An endpoint dilution titration of the ferritin and PrP^{res} peaks has been incubating for 200 days post inoculation and so far supports the incubation time bioassay result. As with most of our other endpoint dilution titrations, these animals will be observed for 540 days (eighteen months) to obtain the highest quality data. It is unlikely, however, that enough animals will develop disease to change the conclusion drawn from the data that most of the ferritin is irrelevant to infectivity.

To our knowledge, no preparation of PrP^{res} has ever been free of ferritin, so the possibility remains that a minority of ferritin or apoferritin is associated with infectivity or PrP^{res}, either *in vivo* or *in vitro*. Further purification is underway to remove the residual ferritin. When this is accomplished, a bioassay will test whether infectivity has been preserved.

Knowing the density range of infectivity in sucrose has already been useful in the development of the step gradient purification protocol described in Appendix A.

Sedimentation Constant

We have also developed and carried out a sucrose gradient sedimentation velocity ultracentrifugation experiment that separates particles based on size, mass, density, and shape. We adjusted the parameters so that PrP^{res} would migrate to the middle of the centrifuge tube during the run. Fractions from a run were inoculated into animals for the bioassay of scrapie infectivity and were tested for PrP^{res} by western blot. The western blots revealed a broad peak of PrP^{res} in the center of the gradient with much lower levels above and below. The bioassay detected very low levels of infectivity. In this low range, the incubation time assay is not as precise as with higher concentrations and the uncertainty in each data point is high, about one log plus or minus. The only definite conclusions that can be made are that infectivity is low at the top and bottom of the gradient and has a large plateau in the middle. It is reasonable to summarize the data by saying that it roughly corresponds to the pattern of PrP^{res}. There is some indication that the more slowly sedimenting particles near the top of the gradient, presumably smaller ones, have a higher specific infectivity relative to PrP^{res}, but this can not be definitely stated. For comparison to PrP^{res} and infectivity, we have characterized the sedimentation behavior of three marker particles with known sedimentation constants. Two viruses, PhiX174 and PCV1, and one proteinaceous particle, apoferritin, were run in parallel gradients and their locations in the fractions determined, using three different laboratory techniques (Figure 5). We are thus able to plot a standard curve using data from these characterized markers and plot the experimental particles' locations against the curve (Figure 5). The PrP^{res} in the inoculated gradient had a heterogeneous sedimentation constant with values in the range of 50-70 Svedbergs (S), which overlapped with the sedimentation constant of PCV1, 52 S.

As with buoyant density, the finding of heterogeneity in the physical properties of PrP^{res} is not surprising. Sedimentation constant ranges of 40 S to 10,000 S have been reported using less purified material. What stands out about our nanofiltrate is the relatively narrow range of 50-70 S. In terms of the prion hypothesis, the heterogeneity can be explained by the various lengths of PrP^{res} fibrils and different numbers of fibrils bundled together.

Knowledge of the sedimentation constant range of PrP^{res} and infectivity in the nanofiltrate influenced our development of the optimized Step Gradient Two protocol described below and is currently being used to design a final purification step to remove residual ferritin from highly purified PrP^{res}.

Aim II. To obtain a monodisperse preparation of TSE infectivity from 10% brain homogenate instead of 1% homogenate, thereby increasing the infectivity of the preparation by a factor of ten.

More Concentrated Preparations

In pursuit of Aim II, we conducted trials with more concentrated brain homogenates to assess their filterability and clarity following filtration. We determined that while a 10% brain homogenate was not efficiently filterable in nanofilters, a 4% preparation was usable. Two liters of 4% brain homogenate were pre-filtered with a 0.2 µm depth filter. Endpoint dilution bioassays have confirmed that no infectivity was lost to the pre-filter (Table 4). One liter of this filtrate was passaged through two Asahi "20N" nanofilters with 18.4 nm +/- 2 nm pore size. Endpoint dilution bioassays have shown that two logs of infectivity were lost to the first 18.4 nm filter, but none was lost to the second (Table 4). We have a liter of a new highly infectious monodisperse preparation with an even smaller particle size than the original monodisperse preparation that came through a 35 nm filter.

We have also produced other infectious stocks of large volume, including two liters of 35 nm filtrate prepared from 1% brain homogenate treated with the nuclease Benzonase and treated twice with the protease trypsin. Two liters of 35 nm filtrate were prepared from 3% brain homogenate treated with Benzonase and trypsin. The above stocks have all been titered by endpoint dilution assay and all contain high levels of infectivity (Table 4). Other non-titered stocks have also been prepared. Our continuing collaboration with the Asahi company allows us access to their nanofilters, which are uniquely well suited for our purposes.

Nanofiltration of Purified Fibrils

Exploring a different approach to large-scale purification, we used nanofiltration on PrP fibrils prepared in a Diringer-style protocol and subjected to dispersion. Western blots revealed that about 6% of the PrP^{res} passed through the 35 nm filter. Endpoint dilution titrations verified that only one log of infectivity was lost from the dispersed fibrils to the 35 nm nanofilter (Table 4). This is close to the same filterability as seen with unpurified brain homogenate, although it must be born in mind that some infectivity, perhaps 0.8 log, is lost during the fibril preparation protocol. It may be that an efficient way to purify infectivity is to do the fibril prep first and then disperse and filter, as opposed to our recent method of putting the entire unpurified brain homogenate through the nanofilter and then purifying.

Future Massive Scale Purification

As will be described below, we have made great progress in the purification of small, dispersed, infectious particles of scrapie infectivity, but the concentration, approximately 7-8 logs IU per ml remains much lower than that achieved in PrP^{res} fibril preps, 10-11 logs IU per ml. We are therefore splitting our efforts into two tracks with the first continuing the purification of PrP^{res} derived from nanofiltrate of brain homogenate. This preparation of highly dispersed protease-resistant PrP^{res} is a unique reagent in the TSE field that may have uses beyond our desire to characterize the biochemical composition of the agent. The second pathway is to

purify PrP^{res} and infectivity starting with the product of an improved fibril prep. Our eighteen month bioassays of the filtered and unfiltered fibrils showed that the infectivity associated with these aggregated structures can be dispersed and therefore is not permanently aggregated. It should therefore be possible to take the impressive concentrating and purifying power of the fibril prep and then follow it with our own developed methods of further purification to polish the infectivity to an even higher degree of purity. Specifically, the fibrils will be dispersed and then subjected to continuous sucrose gradient equilibrium ultracentrifugation followed by additional steps if necessary. We have also learned, from other eighteen month bioassays, that micron range filters do not remove any infectivity from dispersed homogenate. We will therefore pre-filter 10% brain homogenate before subjecting it to the fibril prep procedure. The filtration may remove some contaminants and help to disperse the membranes with which infectivity is associated and give a higher yield of PrP^{res} in the pellet after differential ultracentrifugation.

Aim III. To purify the monodisperse infectivity using sedimentation to equilibrium, sedimentation velocity, and other methods as necessary including column chromatography and electrophoresis.

Sucrose Step Gradient

Aim III was to purify infectivity using data developed during our studies. Combining data from preliminary density and sedimentation studies, we developed a three stage sucrose step gradient. After carrying out the experiment with 200 ml of monodisperse preparation, we saw that PrP^{res} was concentrated 30-fold. Non-PrP proteins were reduced greater than 100-fold to below the limit of detection by silver stain. We have completed the endpoint dilution titration of the final product of the step gradient and it shows that infectivity was concentrated more than 60-fold over the starting monodisperse preparation. Before reading this report further, please see Appendix A for further description and discussion of the first step gradient study.

This successful protocol has been optimized for greater recovery and larger scale. The Step Gradient Two protocol is analogous to the Run A of the first step gradient, but achieves a superior separation of PrP^{res} from non-PrP protein and presumably other soluble contaminants, such as nucleic acids, lipids, polysaccharides, and small molecules. This protocol was applied to one liter of 3% brain 35 nm filtrate. A sample gradient is shown in Figure 6 with fractions analyzed by BIO-RAD Silver Stain Plus and anti-PrP western blot. It can be seen that soluble proteins remain near the top of the tube (higher numbered fractions) while ferritin and PrP^{res} sediment to the lower fractions. Endpoint dilution titration of the Step Gradient Two product has reached 300 days post inoculation and so far shows 7 logs IU per ml. As the incubation continues to 540 days, additional animals are likely to develop disease, increasing the estimated titer.

Through a Pilot Project Grant from the Baltimore Research and Education Foundation, we obtained funds for titration by animal bioassay of the Step Gradient Two product. This small grant did not cover any of the costs of production of the stock or its characterization in the laboratory.

The continuous sucrose gradient equilibrium ultracentrifugation experiment described under Aim I is similar to Run B of the first step gradient and constitutes the next stage in the

scaled up purification after the Step Gradient Two method. In Run B of the first step gradient, we observed some separation of ferritin and PrP^{res}, but we were focused on maintaining the highest recovery of PrP^{res}, so we pooled all four bottom fractions, which contained both ferritin and PrP^{res}. In the revised gradient we used higher densities in the sucrose layers and spun the tubes for 42 hours instead of 18 to allow slowly sedimenting small particles of ferritin and PrP^{res} to reach their buoyant densities and the sucrose layers to form a linear gradient (Figure 3). As described above, the PrP^{res} peak was separated from the ferritin peak, although there was still some overlap. It appears that apoferritin and low-iron ferritin share a buoyant density range with PrP^{res}, as do some other contaminants.

Although the continuous sucrose gradient removed most of the major contaminant, ferritin, the pool of fractions from the continuous gradient was not as pure as Pool C from the first step gradient, in terms of non-PrP, non-ferritin protein. We attempted to use a final step gradient, similar to Run C described in Appendix A, but the purity was not greatly improved. We are currently experimenting with a modified sedimentation velocity ultracentrifugation technique based on the method described above which determined that the peak sedimentation constant range of PrP^{res} in the nanofiltrate is 50-70 S.

PH Study

The infectious agent of the transmissible spongiform encephalopathies has been notoriously difficult to purify in a dispersed form and success in such a purification effort may depend on using every available advantage. Many viral purification procedures are limited to gentle conditions that do not inactivate the virions, but the scrapie agent is resistant to extremes of heat and pH. The possibility exists that an elevated pH may improve the efficiency of a future purification step, such as a density gradient or column chromatography, and allow scrapie infectivity to be concentrated in a dispersed form. A large scale bioassay study of the stability of the scrapie agent to increasing alkalinity has been completed. In our preparation of dispersed small particles, originally prepared at pH 7.2, infectivity is stable (or even enhanced) at pH 9 and reduced by one log at pH 10. Alkalinity of pH 11 or higher eliminates infectivity by more than four logs. Interestingly, we observed that 75% of the PrP^{res} in the dispersed preparation became protease sensitive at pH 9 and 99% at pH 10, values that do not match the effect on infectivity. Unfortunately, the effect of the pH 9 condition of rendering the majority of PrP^{res} sensitive to protease was not reliably reproducible. Because of this and the fact that the effect was not of a high order, even when seen, the approach was not pursued.

Collaborations

With the help of collaborators at the Rocky Mountain Laboratories and the University of Maryland, we have characterized our purified infectious particles by electron microscopy (EM) (see Appendix A), PrP^{C} *in vitro* conversion assay (see Appendix A), and immunofluorescence with anti-PrP antibodies. The EM revealed thin fibrillar structures, presumably PrP^{res}, and a high concentration of iron-loaded ferritin. Our particles were found to have the same in vitro converting activity as a scrapie brain homogenate of higher PrP concentration, attesting to their quality. Our collaborators are using immunofluorescence to explore the structure of synthetic and natural PrP fibrils. Attempts to capture the first images of infectious PrP^{sc} by atomic force microscopy were

dropped because the small, dispersed particles in our purified preparations are too small for the technique. Large aggregates of PrP^{sc} fibrils may be suitable for this method, but they have not been the focus of this project, which is pursuing the elemental form of the infectious agent.

Infectivity of PrP^{sc} that is Protease-Sensitive

Recent reports in the literature claim to study a form of disease-specific PrP oligomer that is infectious and yet protease-sensitive. Most prion literature equates protease-resistance with the infectious character of PrP^{sc}, so these claims challenge the basic concept of what the prion is. In the course of our work, we have conducted three sets of bioassays before and after digestion with the protease trypsin, which is less active than Proteinase K, commonly used to digest scrapie brain homogenate samples (Table 5). Our data shows no loss of infectivity after digestion and, in fact, repeatedly indicates a slight increase, although not greater than the uncertainty in the measurements. Our results is in line with decades old data from bioassays conducted on brain homogenate or PrP fibrils before and after digestion with Proteinase K, which also did not show any significant loss of infectivity. Because most infectivity assays in the TSE literature are done by the less accurate incubation time bioassay and ours are all endpoint dilution titrations, we are considering presenting our titrations as a short paper arguing against the existence of protease-sensitive infectivity. It seems most likely to us that the protease-sensitive PrP that co-purifies with PrP^{res} is PrP^{C} that is physically associated with the PrP^{res}, but has not fully converted to the B-sheet form. It must be kept in mind, however that bioassay results have an uncertainty of at least 0.3 logs, which leaves open the possibility that protease-sensitive PrP^{sc} has a low level of infectivity.

Aim IV. To measure the filtration pore size limit of the TSE infectivity and of the PrP^{res} amyloid in the monodisperse preparation.

Aim IV has been accomplished in collaboration with the Asahi company. Along with the commercially available 35 nm, 24 nm, 18.4 nm, and 15 nm filters, Asahi constructed special 9 nm +/- 4 nm filters for our use (Figure 7). Parallel and serial filtrations were carried out which showed, surprisingly, that scrapie infectivity is capable of passing through the smallest size filters. Because PCV1 was blocked by the 9 nm filter, we now know that the most elemental forms of scrapie infectivity are smaller than the smallest known virus.

We would like to further validate the pore size of the 9 nm filters by assaying the filtrates for hamster ferritin, a natural component of brain homogenate. Ferritin is an iron storage protein that assembles into a hollow sphere consisting of 24 protein subunits, surrounding a crystalline iron-oxygen core. The ferritin particle is 13 nm in diameter and theoretically should pass the 15 nm filter, but not the 9 nm filter. If we can demonstrate the absence of ferritin in the 9 nm filtrate, we will be able to support the claim that TSE infectivity can be smaller than 13 nm.

We have three methods available for development that might be sensitive enough to rigorously demonstrate a lack of ferritin in the 9 nm filtrate and we have attempted to use two of them unsuccessfully. The first method is SDS-PAGE followed by silver stain, but his technique lacked sufficient sensitivity. We developed a western blot using an antibody provided by a collaborator that is specific for mouse ferritin and was verified to cross-react with hamster ferritin. Again the sensitivity was too low and it barely detected endogenous hamster ferritin in the 35 nm filtrate. The 9 nm filtrate had less signal, but it may have been only two or three-fold

less, because the signal in the 35 nm filtrate was so close to the limit of detection. We still have the option to use electron microscopy because the iron core of ferritin is extremely electron dense and easily stands out in a micrograph. For all three methods it is possible that acetone or methanol precipitation of proteins may concentrate the ferritin signal and bring it far enough above the limit of detection that we can claim a significant reduction in the 9 nm filtrate, if such is the case.

New Aim I. To prepare a large stock of purified infectious particles from one liter of infected brain homogenate.

See the discussion above of the Step Gradient Two protocol under original Aim III.

One liter of 35 nm filtrate of 3% brain homogenate was processed through the Step Gradient Two method, resulting in 250 ml of partially purified particles. Separate funding was acquired from the Baltimore Research and Education Foundation to conduct a bioassay of the material. Although incomplete, this bioassay indicates a level of infectivity in the purified material equal to or higher than the starting material, thus validating the scaled up method (Table 6).

New Aim II. To characterize the molecular components of the purified infectivity including proteins, lipids, polysaccharides, and nucleic acids.

Further Proof that the Silver Staining Contaminant (SSC) is Ferritin

Pool C, the product of the first step gradient procedure, (described in Appendix A) contained a component that we have referred to in previous reports to the NPRP as Silver Staining Contaminant (SSC). Appendix A, a manuscript nearly ready for submission, contains evidence that SSC is the iron storage protein ferritin, in its oligomeric state, loaded with iron. Additional evidence comes from western blots using anti-mouse ferritin primary antibodies (Figure 4). Denatured PrP^{res} transfers from an SDS-PAGE gel onto PVDF membrane and is stained by anti-PrP antibody, but not anti-ferritin antibody. In line with expectations, SSC is able to transfer out of a low acrylamide density area of an SDS-PAGE gel onto membrane and is stained by anti-ferritin, but not anti-PrP.

Proteasomes and other non-PrP, non-ferritin components

The presence of proteasomes in the purified preparation was supported by preliminary work with an antibody specific for a conserved region in alpha subunits of the proteasome. This experiment has not been repeated, but when samples of PrP^{res} of even higher purity are available, they will be assayed for proteasomes. If the proteins of the proteasome repeatedly co-purify with PrP^{res}, we will develop a protocol specifically for the purpose of separating them from PrP^{res} as we did with ferritin. If, unlike ferritin, it is impossible to separate the proteasomes from PrP^{res} without affecting infectivity, then the proteasome, also called the Multi-Catalytic Proteinase and Prosome, would be a candidate component of the agent. This outlines the basic approach to all potential non-PrP components. We will also examine the stoichiometry of contaminants to see if they are present at a concentration of at least one unit per infectious dose, which is a requirement for them to be

essential components.

Our collaborator Suzette Priola, Chief of the TSE/Prion Molecular Biology Section at the Rocky Mountain Laboratories, NIAID, NIH is willing to test our purified fractions for non-protein, non-nucleic acid components, such as lipids and polysaccharides. She has access to advanced mass spectrometers and is willing to expose them to scrapie-derived samples. In the past, the unwillingness of core facilities to test TSE samples has been an impediment to research into the biochemistry of infectivity.

Future Massive Scale Purification

See the discussion under original Aim II above of combining an improved fibril prep method with our continuous sucrose gradient equilibrium ultracentrifugation technique. We have already completed preliminary work on the micron range filtration of 10% brain homogenate and verified that, after dispersion, it is filterable and no PrP^{res} is lost to the filter. The next steps will be to experiment with differential ultracentrifugation, which is the basis of the fibril prep. It is expected that we will achieve partial purification of PrP^{res} and also have a more concentrated product that can then be applied to the sucrose gradient and any other purification steps used with our small, dispersed infectious particles. The end result should be a sample of highly purified PrP^{res} with infectivity in the range of 11 logs per ml. Production of this material will greatly assist the search for non-PrP components of infectivity and the preparation may find many uses in TSE research.

New Aim III. To validate the extremely small filtration pore size of the TSE infectivity by assaying ferritin levels in the nanofiltrates of monodisperse preparation.

See the discussion above of original Aim IV, which includes results relevant to this new aim.

KEY RESEARCH ACCOMPLISHMENTS:

The Aims referenced below are taken directly from the approved Statement of Work and Revised Statement of Work.

Aim I - To measure the sedimentation constant and the buoyant density of the infectivity in the monodisperse preparation and to compare these values to those of PrP^{res} in the preparation. The values obtained for the infectivity will be used to guide the purification procedures in Aim III.

- Sedimentation constant range of PrP^{res} and scrapie infectivity determined in parallel with three marker particles.
- Buoyant density of PrP^{res} and infectivity measured twice in CsCl and once in sucrose.
- Determined that CsCl, unlike sucrose, can cause aggregation of PrP^{res}. Despite this, there is only a small loss if infectivity detected and this is possibly due to a disaggregating effect of the use of dilution and ultrasonication before inoculation.
- The results of these studies were applied to the purification of infectivity using sucrose step gradients.

Aim II. To obtain a monodisperse preparation of TSE infectivity from 10% brain homogenate instead of 1% homogenate, thereby increasing the infectivity of the preparation by a factor of ten.

- Brain homogenate and dispersant concentrations for dispersed preparations were optimized.
- Six liters of filtered and nanofiltered preparations have been produced and titered by animal bioassay.

Aim III. To purify the monodisperse infectivity using sedimentation to equilibrium, sedimentation velocity, and other methods as necessary including column chromatography and electrophoresis.

- Endpoint dilution titration in the animal bioassay has proved that dispersed and nanofiltered brain homogenate retains its infectivity after four years at -80°C.
- Titrations have verified that no infectivity is lost during digestion of the nanofiltrate with Benzonase Nuclease and the protease trypsin.
- First Sucrose Step Gradient study concentrated PrP^{res} and infectivity, while non-PrP protein was reduced by 99%.
- Scaled up Sucrose Step Gradient Two developed. See New Aim I.
- Identified the major contaminant of the step gradient particles (called Silver Staining Contaminant) as ferritin with iron.
- Study of pH stability of PrP^{res} and infectivity completed.

Aim IV. To measure the filtration pore size limit of the TSE infectivity and of the PrP^{res} amyloid in the monodisperse preparation.

- Nanofiltration down to a putative 9 nm pore size has been carried out and completed bioassays of infectivity have shown that some portion of the infectivity is able to pass through the smallest filters.

- Identified ferritin as a validation tool for the 9 nm filters.
- See New Aim III.

New Aim I. To prepare a large stock of purified infectious particles from one liter of infected brain homogenate.

- One liter of 35 nanometer filtrate of 3% brain homogenate was processed through the Step Gradient Two protocol, producing 250 ml of partially purified infectious particles.
- Bioassay endpoint dilution titration of the Step Gradient Two product has validated the method; separate funding for the animal bioassay has been acquired.

New Aim II. To characterize the molecular components of the purified infectivity including proteins, lipids, polysaccharides, and nucleic acids.

- The major contaminant of the dispersed, small, infectious particles was definitively identified as ferritin. The presence of proteasomes was also supported.
- No non-PrP components of the purified preparation have been shown to be essential for infectivity.
- Preliminary work at the scale of ten infected brains validated a new approach to the preparation of much more concentrated dispersed, small infectious particles.

New Aim III. To validate the extremely small filtration pore size of the TSE infectivity by assaying ferritin levels in the nanofiltrates of monodisperse preparation.

- Identified endogenous hamster ferritin as a 13 nm particle that should have been blocked by the 9 nm filter. An assay for ferritin could validate the 9 nm filters' average pore diameter.
- Attempted to use PK digestion and silver stain, but the limit of detection was too high.
- Attempted to use a western blot specific for ferritin, but again the sensitivity was too low.
- It is still possible to purify and concentrate ferritin from the nanofiltrate samples and then use one of the above methods or electron microscopy to assay for ferritin.

REPORTABLE OUTCOMES:

- CHI Conference Poster 2007
- VA Research Day Posters 2005, 2006, 2007
- NPRP Conference Presentation 2005
- Manuscript describing the Sucrose Step Gradient nearly ready to submit, see Appendix A.
- Awarded Pilot Project Grant from the Baltimore Research and Education Foundation, Inc.
- Applied for VA Career Development Award.

CONCLUSION:

The goal of this project has been to purify small, dispersed, infectious particles of scrapie infectivity and characterize their components, whether purely protein or containing other substances. We have developed a protocol that purifies PrP^{res} and scrapie infectivity from nanofiltrate of dispersed scrapie brain homogenate. We have continued to improve the purity of the preparation by removing most of the major contaminant, ferritin. We have not identified any non-PrP components of the infectious agent and have not separated infectivity from PrP^{res}. Although some of our fractions have possessed greater specific infectivities relative to PrP^{res} than the starting brain homogenates, our work has generally supported the prion hypothesis that PrP^{res} is the major component of the infectious agent. Minor components may be present at low stoichiometric ratios to PrP. Candidates mentioned in the literature are glycosaminoglycans, lipids, and a polysaccharide similar to glycogen. Because these materials are not as easily detected as proteins or nucleic acids, the only way to test whether they co-purify with infectivity is to create a large scale preparation at a level of purity higher than previously achieved. Our work with fibril preparations, microfiltration, nanofiltration, and the purification of dispersed particles of PrP^{res} has enabled us to design a protocol to accomplish this goal. This work is currently in progress. The identification of a non-PrP component would provide new targets for the development of diagnostics and treatments for these incurable, fatal neurological diseases of animals and humans.

The purified preparations of small, dispersed infectious particles described here may find uses other than biochemical characterization. They can be labeled and tracked to investigate the molecular pathogenesis of TSE infection in cell culture. Another possible use is as a spike in inactivation/removal studies, such as with human blood products. We are pursuing collaborations to exploit uses for the purified particles.

REFERENCES:
See Appendix A.

APPENDICES:
Appendix A: Manuscript “Purification of Highly Dispersed PrP^{res} with PrP^{C} Converting Activity and Scrapie Infectivity” that is nearly ready for submission.

LIST OF PERSONNEL SUPPORTED BY THIS AWARD

1. Sean Carter
2. Luisa Gregori, Ph.D.
3. Michael L. Jackson
4. Jermaine Motse
5. Robert G. Rohwer, Ph.D.
6. Andrew Timmes, Ph.D.
7. Eric Wester
8. Kiesha Wilson

SUPPORTING DATA:

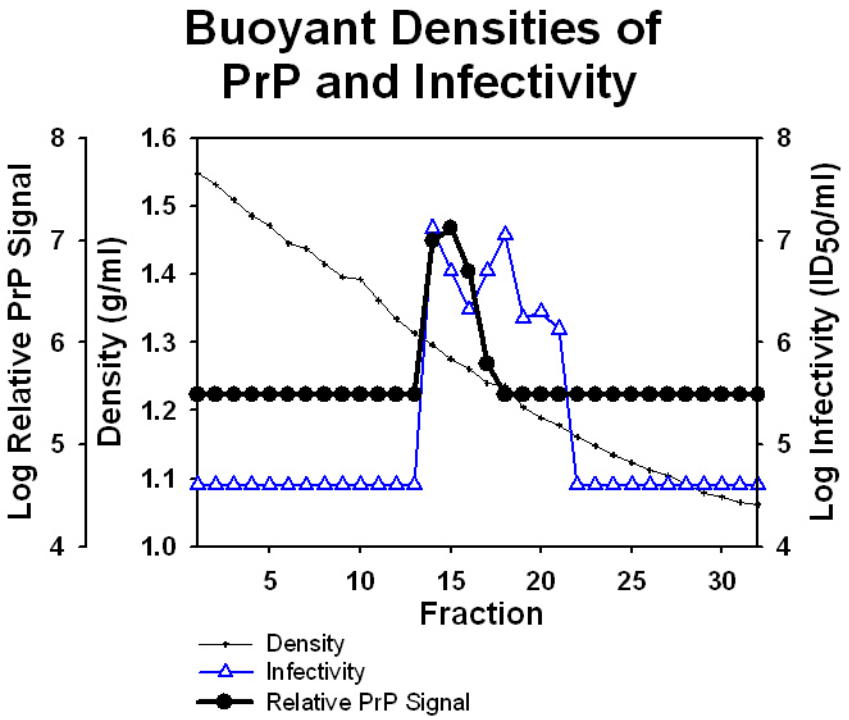


Figure 1. In the new top-loaded CsCl Gradient, PrP^{res} was concentrated into one peak while scrapie infectivity was found in two peaks with a significant shoulder on the lighter peak. 98% of the detectible PrP was in one-tenth of the total gradient. The PrP peak density was 1.28 ± 0.01 g/ml. Infectivity had peak densities of 1.23 and 1.29 ± 0.01 g/ml. The limit of detection for PrP was a 1:43 dilution of the peak signal and all fractions without detectible PrP are plotted at that level. The limit of detection for the incubation time bioassay is 10 ID_{50}/inoculation, which consistently yields infection endpoints before 180 days post inoculation. Considering the dilution of the inoculum and the volume inoculated, our limit of detection was 40,000 ID_{50}/ml. All fractions with less than 10 ID_{50}/inoculation have been plotted at this limit.

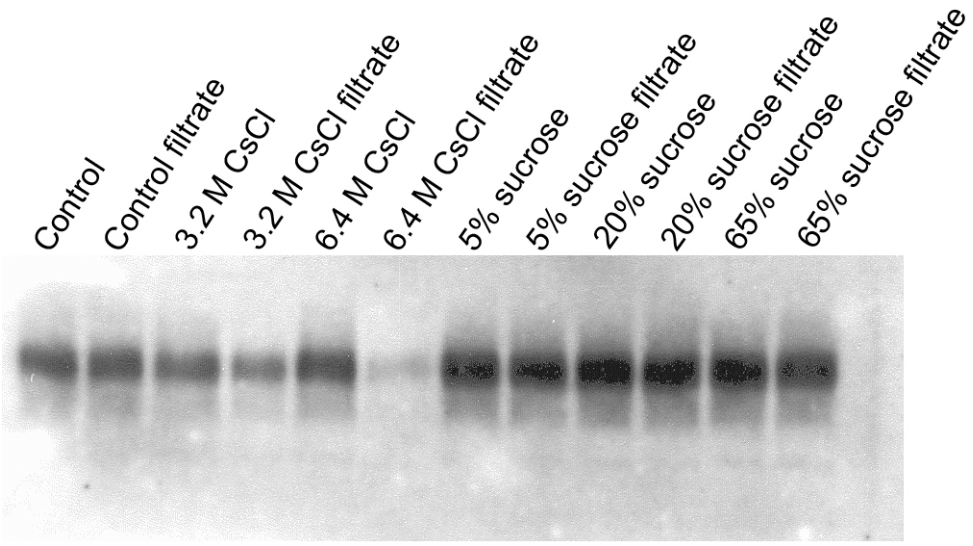
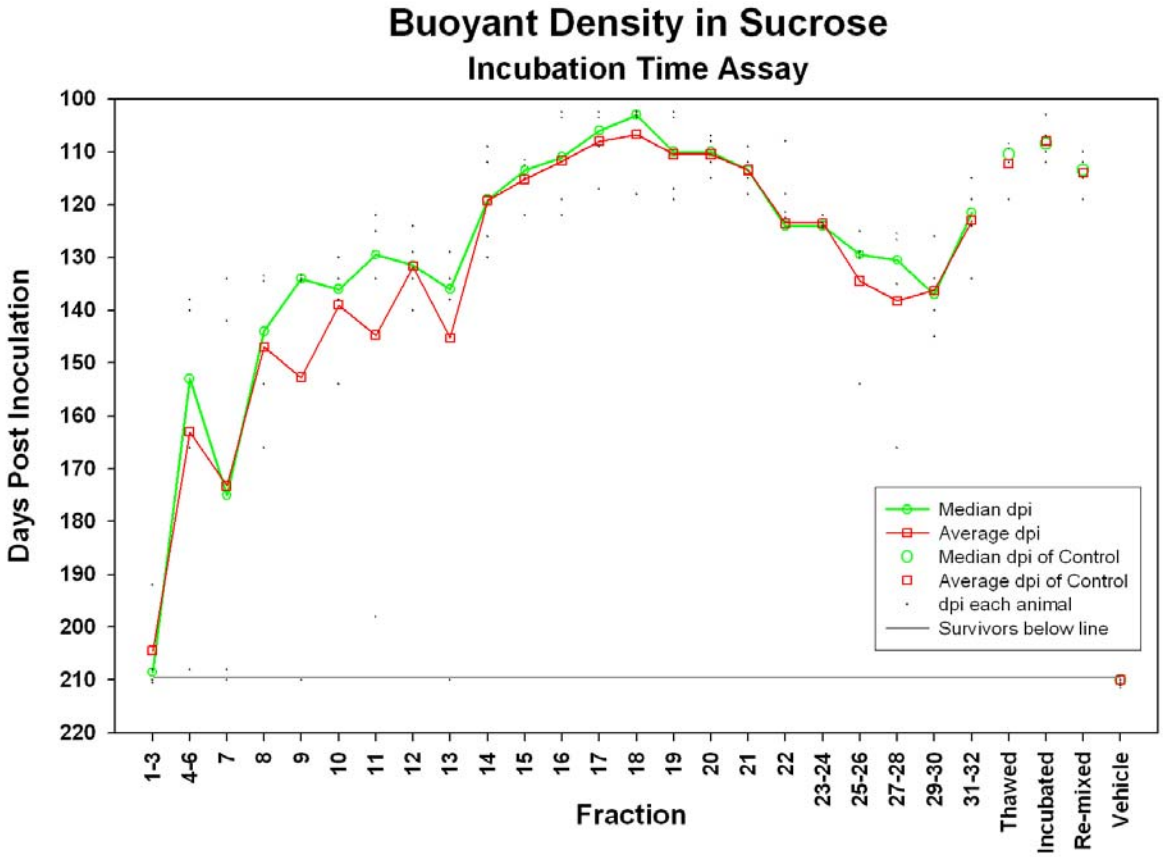


Figure 2. A high concentration of CsCl induces aggregation of PrP^{res} from the monodisperse preparation. Following incubation with CsCl, PrP^{res} that previously passed through a 35 nm pore size nanofilter was unable to penetrate a 220 nm filter. Sucrose did not cause a loss to the filter. The average particle size of PrP^{res} increases upon incubation in CsCl.



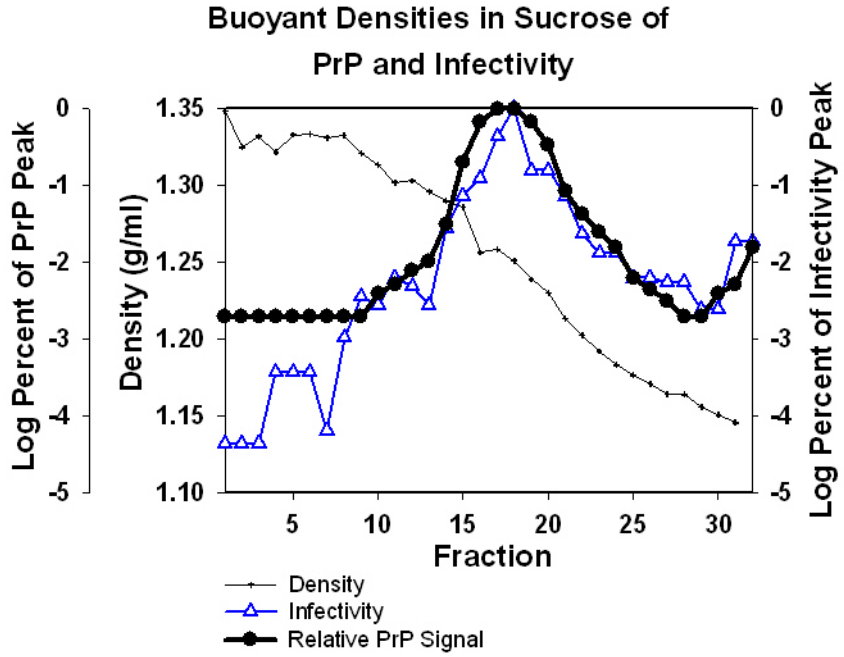


Figure 3. See next page for legend.

Figure 3. See previous page for images. Infectivity and PrP^{res} were concentrated together by the ultracentrifugation. The top figure shows fractions or fraction pools versus days post inoculation (dpi) at death from scrapie. Animals inoculated with fractions in the middle of the gradient died earlier, which according to the dose-response of scrapie infection means that they received higher dosage. Both the medians and averages are shown as well as dots for each animal. Animals which survived to the end of the experiment are indicated by dots below the gray line. The first control was starting material thawed from frozen storage and immediately inoculated. The second was material incubated at ambient temperature for 42 hours while the ultracentrifuge was running. The re-mixed control tested recovery from the ultracentrifuge tube; equal portions from every fraction were combined before the mixture was inoculated. The controls all had the expected high levels of infectivity and can be compared to the peak fractions of the gradient to show that recovery of infectivity was good.

The bottom figure has a plot of incubation time data (dpi) converted to titer by a dose-response curve developed in our laboratory. The titers were then normalized to the fraction with the highest value and plotted on a log scale. PrP western blot signal was also converted to percentage of the PrP peak signal and plotted on the same scale for direct comparison. The two data sets coincide within the error of the bioassay demonstrating co-purification of PrP^{res} and infectivity. The density of each fraction was measured gravimetrically and is plotted on the same graph.

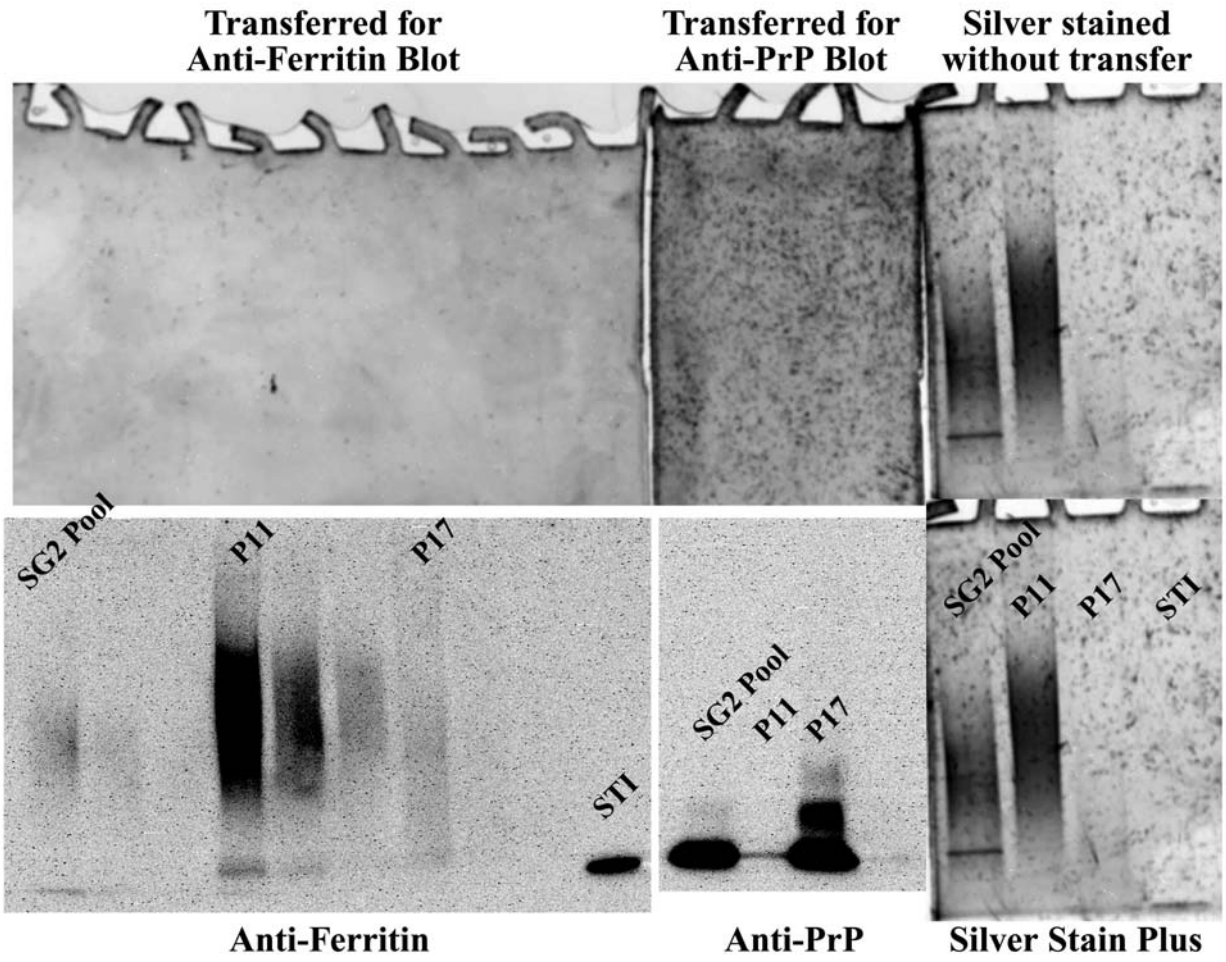
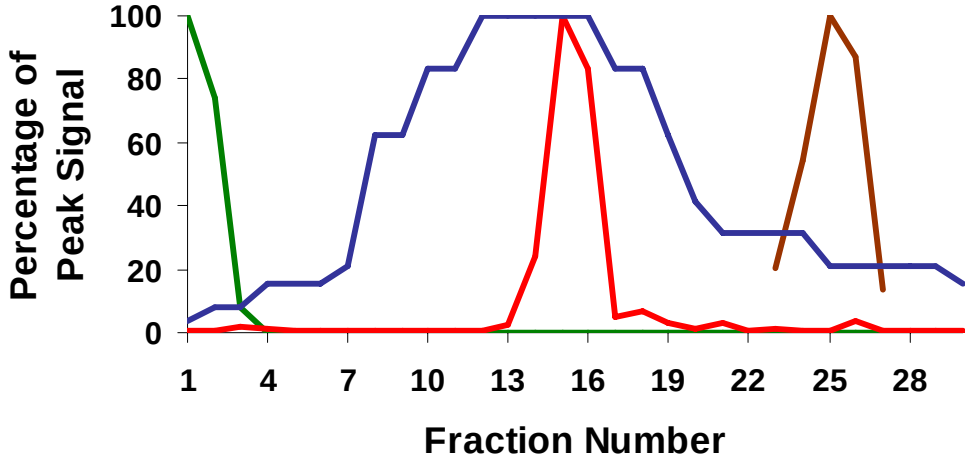
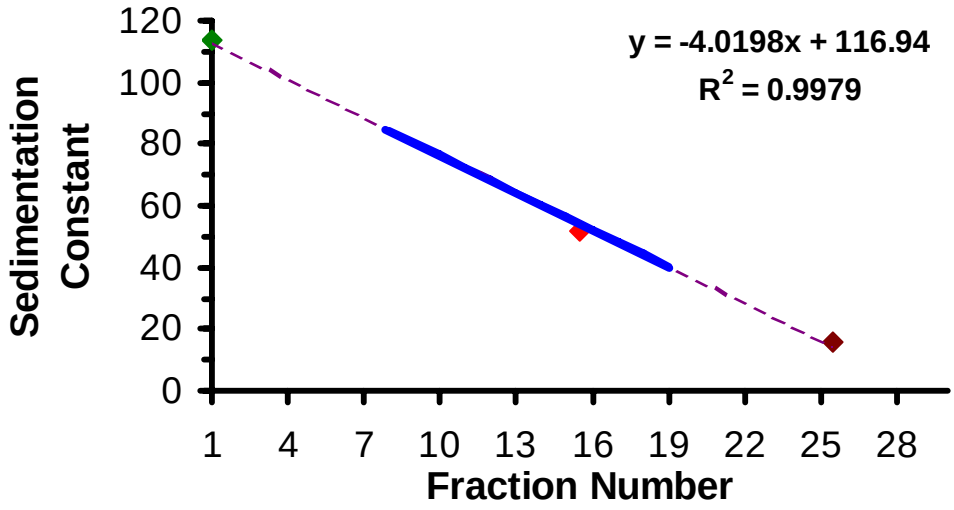


Figure 4. Samples containing ferritin and PrP^{res} were loaded onto three sections of an acrylamide gradient gel for analysis by three methods, Ferritin western blot, PrP western blot, and silver stain. Fractions P11 and P17 contain the separated ferritin and PrP^{res}, respectively, from the SG2 Pool preparation, which is partially purified from scrapie brain homogenate. The left section was loaded with each sample undiluted, diluted 1:3, and diluted 1:9. This section was subjected to western transfer and was stained with anti-ferritin antibody. The rightmost lane of this section contained the molecular weight (MW) standards Soybean Trypsin Inhibitor (STI) and Carbonic Anhydrase (CA), each loaded at 100 ng per lane. The STI cross-reacted with the anti-ferritin antibody 5B5. P11, which has the most silver staining component, shows the most signal here. Both a high MW smear from oligomers and a thin band of ferritin subunits at the dye front can be seen. The middle section was loaded with undiluted samples and the MW standards, western transferred, and stained with anti-PrP antibody 3F4. P11 has very little PrP signal while SG2 Pool and P17 show high levels. The usual PrP glycoform pattern is not seen because electrophoresis was stopped before the three glycoforms could separate. This was done to prevent any ferritin from migrating into dense acrylamide, where it would be trapped. Higher MW forms easily seen in P17 are faintly visible in SG2 Pool, which has less total PrP. The right section was loaded with undiluted samples and was not transferred. The silver staining component seen in the right gel section and transferred out of the other two sections was not stained with anti-PrP antibody, but did stain with anti-ferritin antibody, establishing its identity as ferritin.
Order on gel: SG2 Pool undiluted, 1:3, 1:9; P11 undiluted, 1:3, 1:9; P17 undiluted, 1:3, 1:9; STI/CA; SG2 Pool, P11, P17; STI/CA; SG2 Pool, P11, P17; STI/CA
STI and CA were loaded together at 100 ng per lane





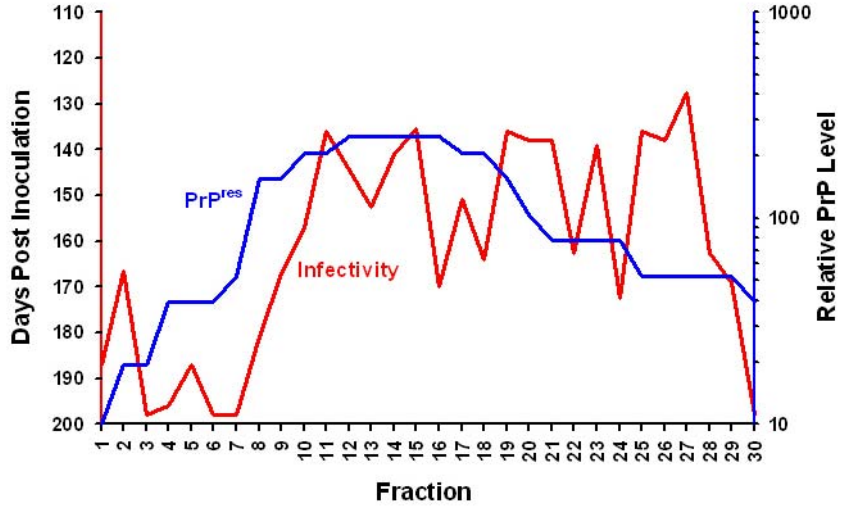


Figure 5. See next page for legend.

Figure 5. See previous page for images. As plotted in the top figure, PrP^{res} (blue) was

predominantly found in fractions 8-19, with a broad peak between fractions 12-16, corresponding to 50 to 70 Svedbergs (S). The marker particles with known constants were found in the following fractions: PhiX174 (green), 114 S, fractions 1 and 2; PCV1 (red), 52 S, fractions 15 and 16; Apoferritin (brown), 18 S, fraction 26. In the top plot, each marker is plotted as a percentage of its own peak signal so that they are all normalized to a peak height of 100%. The middle figure shows a standard curve generated by plotting the marker particles' positions, which allowed us to interpolate the sedimentation constant range of PrP^{res}. The bottom figure compares a plot of average days post inoculation at death from scrapie and PrP western blot signal on a log scale. The scales are not directly comparable, but the figure shows that both are low at the bottom of the gradient (low number fractions) and high in the middle. While both are low in the uppermost fractions (where the sample was loaded), there is an indication that some fractions near the top of the gradient may have a higher specific infectivity relative to mass of PrP^{res}.

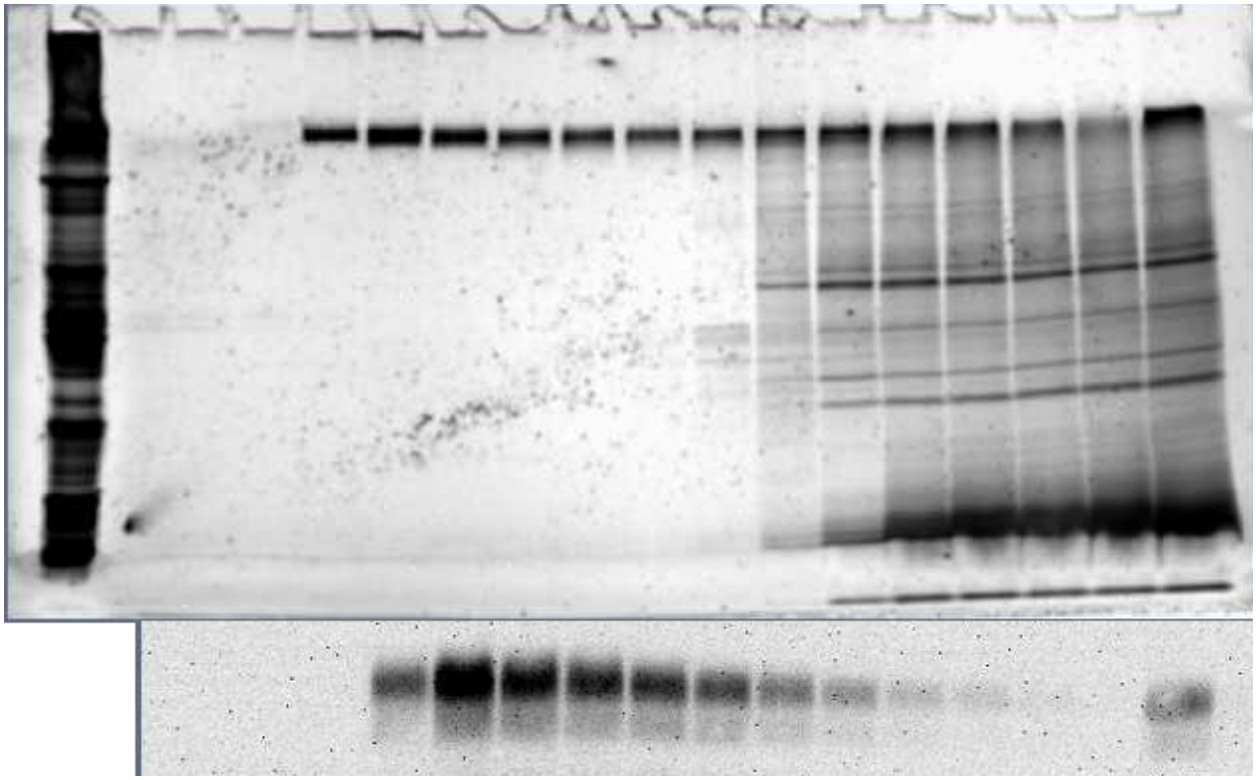


Figure 6. Fractions from the scaled up sucrose step gradient were examined by BIO-RAD Silver Stain Plus kit (top image) and western blot with 3F4 antibody (bottom image). Left to right: Broad Range MW Std, Fractions 1-15, Combined fractions 16-30, Unspun starting material (3% SBH 35 nm filtrate treated with Benzonase and trypsin). Sample began the run at the location of fractions 14 to 30. During the run, PrP^{res} sedimented into the sucrose layers as far as fraction 5, while soluble protein only penetrated as far as fraction 10. The silver stained top image reveals that the silver staining contaminant, identified as ferritin, sedimented farther into the dense sucrose cushion than PrP^{res}. Lane 17 shows that SSC is depleted from the top half of the centrifuge tube (because so much of it sedimented into the bottom half).

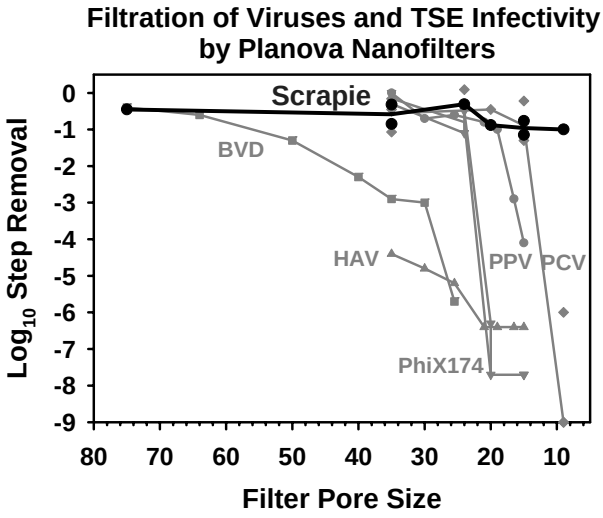


Figure 7. When dispersed scrapie brain homogenate was passed through an Asahi 75 nm filter, there was no retention of infectivity. Moreover, the 75 nm filtrate passed through a 35 nm filter with negligible loss of infectivity indicating a particle size smaller than 35 nm. The 35 nm filtrate in turn passed, with only non-specific losses, through 24 nm, 18.4 nm, and 15 nm filters. A PhiX174 phage control passed the 24 nm filter but was completely blocked by the 18.4 nm filter consistent with its 24 nm diameter. A porcine circovirus (PCV) control passed all filters with an efficiency comparable to the scrapie infectivity. PCV is the smallest known virus of which we are aware. Subsequent passage through a nominal 9 nm filter custom built for us by Asahi completely blocked PCV but continued to pass TSE infectivity. This shows that the size of the elemental particle is smaller than the smallest known virus, PCV, with a 17 nm diameter as measured by electron microscopy. We have not yet been able to validate the pore size of the nominal 9 nm filter, but hope that examinations of the ferritin content of the filtrates will accomplish this goal. Ferritin, an endogenous component of hamster brain homogenate, has a diameter of 13 nm and should have been blocked by the 9 nm filter.

Fraction	Log_{10} ID_{50}/ml so far	Log Dilution Factors (5×10^{x})					
		-3	-4	-5	-6	-7	-8
14	6.7	5/5*	4/4	4/4	4/8	1/7	0/4
16	5.7	4/4	4/4	4/8	1/8	0/8	0/4
18	6.1	4/4	4/4	4/4	0/8	0/8	0/4

Table 1. Fractions from a continuous CsCl gradient equilibrium ultracentrifigation experiment were inoculated for endpoint dilution titration. The fractions were previously tested by the incubation time bioassay , which is a less accurate method. Fraction 14 contains the PrP^{res} peak and had high infectivity by the incubation time method. Fraction 18 did not contain a PrP^{res} peak, but did show an infectivity peak in the first bioassay. Fraction 16 is the trough between the two infectivity peaks. At 300 days post inoculation (dpi), the Reed-Muench calculated titers are 6.7, 5.7, and 6.1 log_{10} ID_{50} per ml. Our standard length of time for titrations is 18 months, or 540 dpi, so there is still much time for additional animals to develop disease and increase the calculated titers. According to our experience, it is unlikely that the final titer will change by more than 0.3 log after 300 dpi. Dilution factors are 5×10^{-3}, 5×10^{-4}, etc.
*Numbers are those that developed scrapie over total inoculated.

Substrate	Treatment	$Log_{10}ID_{50}$ /g brain	Titer change $Log_{10}ID_{50}$ /g brain
Monodisperse Preparation	PBS, 10 min	6.8	
Monodisperse Preparation	PBS, 24 hrs	6.8	+/-0
Monodisperse Preparation	CsCl, 10 min	6.8	+/-0
Monodisperse Preparation	CsCl, 24 hrs	6.3	-0.5
Monodisperse Preparation	NaCl, 24 hrs	5.9	-0.9
Scrapie brain homogenate	CsCl, 24 hrs	10	+/-0

Table 2. Incubation of scrapie infectivity with a high concentration of CsCl for twenty-four hours led to no loss of infectivity from scrapie brain homogenate and a small loss from monodisperse preparation. Incubation with 2.5 M NaCl led to a more substantial loss of infectivity, 87%. The control incubation of monodisperse preparation in the same buffer it was made in, PBS with detergent, demonstrated the stability of infectivity. The preservation of infectivity after a ten minute incubation in CsCl confirms that osmotic shock from rapid dilution is not the cause of lost infectivity. High salt concentrations most likely do not cause true inactivation of scrapie infectivity, but can generate an apparent loss of titer.

Fraction	Log_{10} ID_{50}/ml so far	Log Dilution Factors							
		-2	-3	-4	-5	-6	-7	-8	-9
11/12	4.8	4/4*	4/4	0/8	0/8	0/4	0/4	0/0	0/0
17	6.3	4/4	4/4	4/4	2/4	0/8	0/12	0/8	0/4

Table 3. Fractions containing the ferritin and PrP^{res} peaks from a continuous sucrose gradient equilibrium ultracentrifugation experiment were inoculated for endpoint dilution titration. At 215 days post inoculation (dpi), the Reed-Muench calculated titers are 4.8 and 6.3 log_{10} ID_{50} per ml. Our standard length of time for titrations is 18 months, or 540 dpi, so there is still much time for additional animals to develop disease and increase the calculated titers. According to our experience, it is unlikely that the final titer will change by more than 0.3 log after 215 dpi. *Numbers are those that developed scrapie over total inoculated.

Sample	Treatment	$Log_{10}ID_{50}$/ml	Titer change $Log_{10}ID_{50}$/ml
4% brain homog	Unfiltered	8.1	
4% brain homog	0.2 μm filter	8.5	+0.4
4% brain homog	1st 20N filter	6.6	-1.9
4% brain homog	2nd 20N filter	6.6	+/-0
3% brain homog	35 nm filter	6.8	
3% brain homog	35 nm and trypsin	7.4	+0.6
1% brain homog	75, 35 nm filters	6.9	
Fibril Prep	Unfiltered	10.5	
Fibril Prep	divided by 400	7.9	
Fibril Prep	35 nm filter	7.0	-0.9

Table 4. Approximately two logs of infectivity are lost by passage of 4% dispersed scrapie brain homogenate through an Asahi Planova 20N (18.4 nm ± 2 nm) filter. No significant change in infectivity was detected after passage through the 0.2 μm pre-filter or a second serial 20N filter. In fact, the 0.2 μm filtrate is actually 0.4 logs higher in apparent titer than the unfiltered homogenate. This increase is possibly due to the filter breaking up membranes with which the PrP^{res} is associated, but each measurement has an uncertainty of +/- 0.48 log_{10}, so the difference is not really statistically significant.

Sample	Treatment	$Log_{10}ID_{50}$/ml	Titer change $Log_{10}ID_{50}$/ml
1% brain homog	35 nm filter	7.0	
1% brain homog	35 nm and trypsin	7.8	+0.8
1% brain homog	35 nm and trypsin	7.1	+0.1
1% brain homog	35 nm filter, pH 10	5.9	
1% brain homog	35 nm, pH 10, trypsin	6.0	+0.1
3% brain homog	35 nm filter	6.8	
3% brain homog	35 nm and trypsin	7.4	+0.6

Table 5. Trypsin does not reduce total infectivity in three sets of samples tested by endpoint dilution titrations before and after digestion. 35 nm Filtrate has been titered nine times with an average titer of 7.0 logs ID_{50} per ml. A sample was digested with trypsin and immediately titered, resulting in a value of 7.8 logs ID_{50} per ml. This same sample was thawed from frozen storage after eighteen months and re-inoculated for another 18 month endpoint dilution titration with more dilutions and animals for greater accuracy, resulting in a value of 7.1 ID_{50} per ml. The difference of 0.7 logs between two measurements of the same sample is unusual. It seems most likely that the infections leading to the 7.8 value were outliers or the sample actually lost infectivity during storage, although the undigested 35 nm Filtrate has not lost infectivity after four years in storage.

35 nm Filtrate was adjusted to pH 9.0 with Borate buffer and NaOH and incubated for 24 hours. An aliquot was inoculated for endpoint dilution titration and another was digested with trypsin before inoculation. The titer changed by an insignificant +0.1 log after digestion.

A different 35 nm Filtrate made from 3% brain homogenate was also titered before and after treatment with trypsin. This time the titer increased 0.6 log.

Log_{10} ID_{50}/ml	Log Dilution Factors							
so far	-2	-3	-4	-5	-6	-7	-8	-9
7.0	4/4*	4/4	4/4	4/4	2/8	0/12	0/8	0/4

Table 6. Step Gradient Two Pool, the large scale product of partially purified small, dispersed, infectious particles was inoculated for endpoint dilution titration. At 300 days post inoculation (dpi), the Reed-Muench calculated titer is 7.0 log_{10} ID_{50} per ml. Our standard length of time for titrations is 18 months, or 540 dpi, so there is still much time for additional animals to develop disease and increase the calculated titer. According to our experience, it is unlikely that the final titer will change by more than 0.3 log after 300 dpi.
*Numbers are those that developed scrapie over total inoculated.